DETERMINATION OF NANOSECOND RADIATIVE LIFETIMES OF FLUORESCENCE IN SOLUTIONS WITH THE AID OF A PHASE FLUOROMETER

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Calibrating tests and measurements were made with a new phase fluorometer developed on base of earlier similar instruments, enabling to measure nsec fluorescence decay times (from 0,1—30 nsec) with an absolute error of about $\pm 0{,}07$ nsec. The influence of secondary luminescence on decay time has been studied experimentally and some examples of the importance of this effect are presented. By a relatively simple method of determining the true decay time τ — applicable under workable experimental conditions — the tedious method of correction for secondary luminescence based on further luminescence characteristics can be avoided.

1. Among the quantities characterising the photoluminescence in solutions the decay time τ of fluorescence, *i.e.* the mean lifetime of the excited state, plays an important role in dealing with the mechanism and kinetics of luminescence. The lifetime is closely connected with other luminescence characteristics, as with the quantum yield, the degree of polarisation, with the absorption and emission spectra, respectively the area below the absorption spectrum.

The great deviations among decay times measured by different investigators with the same dyestuff are mainly due to secondary luminescence, in consequence of the various methods of excitation and observation as well as to the differences in concentration and layer thickness employed. This fact was pointed out earlier by some authors, but BUDÓ and SZALAY [1] were the first to make exact investigations about the influence of secondary luminescence, demonstrating that the decay time is substantially altered by secondary luminescence due to reabsorption of the primary fluorescence light.

Since the factors mentioned above are mostly lacking or inadequately referred to in the considerable quantity of data about τ available in literature, furthermore the conditions of excitation and observation necessary for a relatively simple calculation of the true decay time had not been accomplished, it seemed useful to build an apparatus meeting these requirements.

2. *The phase fluoromèter* constructed by the authors is based essentially upon the same principle as the apparatus described by BAUER and ROZWADOWSKI [2], but several modifications have been made in its construction.

Spectrum lines of a high-pressure mercury lamp (HBO 500) isolated by interference filters were used as light source. The unit modulating the exciting light consisted of a piezoelectric quartz crystal fed by a generator of ultrasonic frequency and of a superposed fused quartz cube placed between crossed polaroid filters. This quartz cube becomes double refracting because of the standing waves induced

in it. The frequency of modulation was 10,24 Mc. In order to increase the signal-to-noise ratio a mechanical modulator (chopper) was used. The phase differenc between exciting and luminescence light was measured by changing the optical pat length.

Photomultipliers EMI 9558 A were used in the apparatus. The "mirror curves" were recorded automatically with a recorder of type EPP 09. Excitation and observation were performed on the same face of the cuvette containing the solution, whic rendered possible to fulfil the conditions necessary for the correction of secondar luminescence described in [1]. This was achieved by mirrors and lenses built into a tempered sample holder. The exciting light beam forming an angle of about 20° with the direction of observation perpendicular to the front face of the cuvette, and focussed on the front face of the solution in the cuvette, gave a nearly point-like light spot, while the image of a larger circular area of radius $R=0{,}8$ cm was formed on the cathode of the photomultiplier indicating the fluorescence light. In this way it was possible to measure the decay time of solutions of very small layer thickness ($\approx 10^{-3}$ cm), while *e.g.* in the apparatus described in [2] a cuvette of about 1 cm was used with transversal observation.

The decay time for phase fluorometers is calculated according to the well known equation

$$\operatorname{tg}\varphi=\omega\tau, \tag{2,1}$$

where φ is the phase difference between fluorescence and exciting light, and ω is the angular frequency of modulation. If the approximation $\operatorname{tg}\varphi\approx\varphi$ can be used, then

$$\tau=\frac{2\Delta L}{c}, \tag{2,2}$$

where c is the velocity of light, ΔL is the difference in optical path length, proportional to the phase difference φ. In our investigations the decay time τ was calculated according to the exact relation (2,1) instead of (2,2) used *e.g.* in [2], because, in case of decay times of ≈ 5—6 nsec, which are characteristic for dyestuff solutions, a difference of about 4—5% was found between the results calculated with both methods. This would mean a systematic error of the same magnitude as the random error of measurement.

3. *Determination of the true decay time.* According to BUDÓ and SZALAY [1] the relation between the true decay time τ and the measured decay time τ' can approximatively be written as follows:

$$\tau=(1-\varkappa)\tau', \tag{3,1}$$

where $\varkappa$ is the quotient of the intensities of primary and secondary fluorescence. The calculation of $\varkappa$ is tedious and it requires the knowledge of the absorption and emission spectra as well as of the quantum yield [3]. Therefore a method for the calculation of the true decay time is suggested, based upon a procedure similar to that employed in [4] for the calculation of the true degree of polarisation, which does not require the knowledge of further luminescence characteristics beyond τ' measured with three different layer thicknesses.

If the solution practically does not absorb light at the wavelength λ' of observation ($\beta=0$), the value of $\varkappa$ for layer thickness l can be approximately written, according to BUDÓ and KETSKEMÉTY [3], as follows:

$$\varkappa=\varkappa(\alpha,\gamma;m)=\int_0^\infty \eta(\lambda'')f(\lambda'')Md\lambda'', \tag{3,2}$$

where M is a function of α, γ and m defined by

$$M=\frac{1}{2}\left\{\gamma\,\mathrm{Ei}(-m\gamma)-\gamma\,\mathrm{Ei}(-\gamma)+\chi(0,\gamma)+\frac{\chi(\alpha,\gamma)+\psi(\alpha,\gamma)}{1-e^{-\alpha}}\right\},$$

with

$$\chi(\alpha,\gamma)=\frac{\gamma}{\alpha}[G(-\gamma)-G(-\gamma-\alpha)],\quad \psi(\alpha,\gamma)=\frac{\gamma e^{-\alpha}}{\alpha}[G(-\gamma)-G(-\gamma+\alpha)],$$

$$G(x)=\mathrm{Ei}(x)-\log|x|,$$

$$\alpha=2{,}30\varepsilon(\lambda)cl=k_\lambda l,\quad \beta=2{,}30\varepsilon(\lambda')cl=k_{\lambda'}l,\quad \gamma=2{,}30\varepsilon(\lambda'')cl=k_{\lambda''}l,\quad \text{and}\quad m=\frac{R}{l}.$$

Here λ is the wavelength of the monochromatic exciting light, λ' the wavelength of the observed part of the fluorescence spectrum, λ'' the variable of integration, namely the wavelength of the observed part of the primary fluorescence light; R is the radius of the circular area observed on the cathode of the photomultiplier in case of punctiform excitation.

By expanding (3,2) according to increasing powers of γ, the following expression can be obtained

$$\varkappa=\frac{1}{2}\left[\overline{\gamma}\left(\log m+\frac{3}{2}\right)-\overline{\gamma^2}\left(m-\frac{1}{3}\right)+\frac{\overline{\gamma^3}}{2.2!}\left(m^2-\frac{1}{6}-\ldots\right)\right], \tag{3,3}$$

where the mean values $\overline{\gamma^n}$ are to be determined by the integration $\overline{\gamma^n}=\int_0^\infty \eta(\lambda'')f(\lambda'')\gamma^n d\lambda''$, ($n=1, 2, 3,$ ...). The dependence of $\varkappa$ on the layer thickness l can be immediately obtained from (3,3) by substituting $m=R/l$ and $\overline{\gamma}=\overline{lk_{\lambda''}}$:

$$\varkappa=l\left(\frac{\overline{k_{\lambda''}}}{2}\log R+\frac{3}{4}\overline{k_{\lambda''}}-\frac{1}{2}\overline{k^2_{\lambda''}}R+\frac{1}{8}\overline{k^3_{\lambda''}}R^2+\ldots\right)-l\log l\cdot\frac{\overline{k_{\lambda''}}}{2}+ \tag{3,4}$$

$$+l^2\frac{\overline{k^2_{\lambda''}}}{6}-\frac{l^3}{48}\overline{k^3_{\lambda''}}+\ldots.$$

For sufficiently small layer thicknesses the terms containing the square and higher powers of l (more exactly, the term averaged over γ^2 and higher powers of γ) in (3,3) can be neglected, which gives:

$$\varkappa=lf_1(R,k_{\lambda''})-l\log l\cdot f_2(k_{\lambda''}), \tag{3,5}$$

where

$$f_1(R,k_{\lambda''})=\frac{\overline{k_{\lambda''}}}{2}\log R+\frac{3}{4}\overline{k_{\lambda''}}-\frac{1}{2}\overline{k^2_{\lambda''}}R+\ldots,\quad f_2(k_{\lambda''})=\frac{\overline{k_{\lambda''}}}{2}.$$

Since, according to [1]

$$\tau=\tau'(1-\varkappa)=\tau'(1-lf_1+l\log l\cdot f_2), \tag{3,6}$$

we obtain

$$\tau'=\frac{\tau}{1-\varkappa}=\frac{\tau}{1-lf_1+l\log l\cdot f_2}, \tag{3,7}$$

and

$$\frac{1}{\tau'}=\frac{1}{\tau}-l\frac{f_1}{\tau}+l\log l\frac{f_2}{\tau}=\frac{1}{\tau}+lF_1+l\lg l\cdot F_2, \tag{3,8}$$

where

$$F_1\left(=F_1(R, k_{\lambda''}, \tau)\right) = -\frac{f_1}{\tau}, \quad F_2\left(=F_2(k_{\lambda''}, \tau)\right) = 2{,}30\,\frac{f_2}{\tau}.$$

As (3,8) contains three variables (τ, F_1, F_2), the true decay time τ can be determined by resolving the following inhomogeneous linear simultaneous equations with three unknowns, for three decay times (τ_1, τ_2, τ_3) measured with different layer thicknesses (l_1, l_2, l_3).

$$\left.\begin{aligned}\frac{1}{\tau_1'} &= \frac{1}{\tau} + l_1F_1 + l_1 \lg l_1 \cdot F_2\\ \frac{1}{\tau_2'} &= \frac{1}{\tau} + l_2F_1 + l_2 \lg l_2 \cdot F_2\\ \frac{1}{\tau_3'} &= \frac{1}{\tau} + l_3F_1 + l_3 \lg l_3 \cdot F_2\end{aligned}\right\}. \tag{3,9}$$

For the true decay time τ we obtain according to Cramer's rule:

$$\tau = \frac{l_2l_3 \lg \frac{l_3}{l_2} + l_3l_1 \lg \frac{l_1}{l_3} + l_1l_2 \lg \frac{l_2}{l_1}}{\frac{1}{\tau_1'} l_2l_3 \lg \frac{l_3}{l_2} + \frac{1}{\tau_2'} l_3l_1 \lg \frac{l_1}{l_3} + \frac{1}{\tau_3'} l_1l_2 \lg \frac{l_2}{l_1}}. \tag{3,10}$$

In consequence of the neglection of the mean values of γ^2 and higher powers of γ, this expression for the true life-time τ is valid within an accuracy of some per cent strictly speaking only for $\gamma_{max}\,(=2{,}30 \cdot \varepsilon_{max}\, cl) < 1$. However, as practical calculations show, this upper limit can generally be extended to $\gamma_{max} \approx 3$ without increasing the error. With regard to the accuracy of calculation it is convenient to choose sufficiently different layer thicknesses l_i between the limits mentioned above, *e.g.* $l_1 \gtrsim 3l_2$, $l_2 \gtrsim 3l_3$.

The correction for secondary luminescence is, of course, to be carried out only for solutions with considerable overlapping absorption and emission spectra.

4. The following *calibrating tests of our fluorometer* have been performed:

Measurement of the degree of modulation. In order to obtain optimal adjustment of the unit modulating the exciting light, according to [5], the parallel light beam of a tungsten lamp, modulated with high and audio frequency by the piezo-quartz unit and the mechanical modulator, fell on the cathode of a photomultiplier type RCA 931A. The induced high and audio frequency photocurrents amplified by a cathode follower were indicated by a high frequency oscilloscope EMG type 1589-U-1. The degree of modulation was determined by the quotient of high and audio frequency amplitudes. The latter is proportional to the intensity of modulated light.

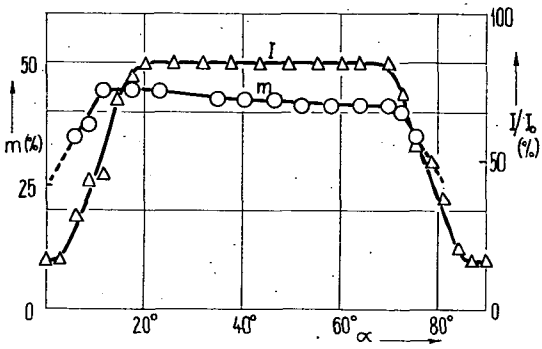

Fig. 1.

In Fig. 1. the degree of modulation in per cent is plotted as a function of the angle α formed by the plane of vibration of the second polaroid filter of the modulating unit and the normal of the ultrasonic standing waves induced in the fused

quartz cube (curve *m*). Curve *I* shows the relative intensity of the modulated light, measured as above, as a function of the angle α. Both curves show broad maxima. This allows to adjust the second polaroid filter with an angle α as low as possible (21°), in order to minimize the losses in exciting light caused by the polaroid placed before the sample holder, serving to adjust the normal of the plane of vibration.

The electron transit times of the photomultipliers, of the order of nsec, depend on the wavelength and intensity of the light impinging on the photo-cathode [6], [9]. Fig. 2 shows the wavelength dependence of the relative variation of the electron transit time of the photomultiplier receiving the luminescence and the scattered exciting light. Since the mean wavelengths of the exciting light and of the observed luminescence light are different, the measured decay times are to be corrected by means of Fig. 2.

The results of measurements made with a scatterer for various light intensities are shown in Fig. 3, in which the relative variation of the electron transit time is plotted as a function of the mean value of the photocurrent, proportional to the light intensity incident on the photomultiplier. Equal intensities of luminescence light and scattered exciting light in measuring τ can be easily obtained with our fluorometer by means of a light reducer (polaroid etc.); so there is no need for correction with the aid of Fig. 3.

The decay time τ' observed directly — especially in case of low intensities of luminescence light — is considerably reduced by the exciting light scattered in the solution, on the wall of the cuvette and other surfaces in the sample holder, and passed by the complementary filter. The correction for this scattered light has been made with the method given in [6]. For this it is necessary to know the ratio of the photocurrents due to scattered exciting light and luminescence light. However, since the signal on the output of our instrument is not proportional to the photocurrent of the photomultiplier, it is important to know the dependence of the mean value I of the photocurrent on the output voltage U_W. This is shown in Fig. 4 in logarithmic scale.

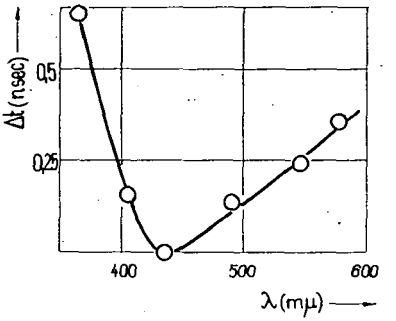

Fig. 2.

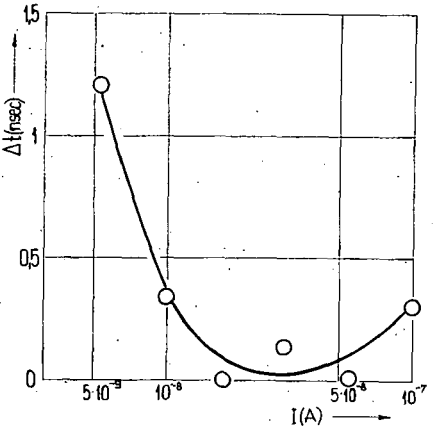

Fig. 3.

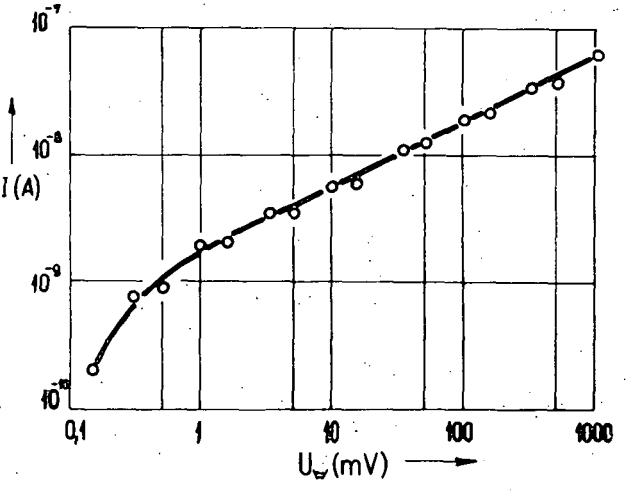

Fig. 4.

5. According to the above, the intensity of secondary luminescence and therefore τ' in a given luminescent solution depend on both the layer thickness l and the concentration c_M of the solution. To illustrate this effect, the decay times of some dyestuff sulutions measured with different layer thicknesses and corrected according to Eq. (3,1) are shown

Table 1/a

1	2	3	4	5	6	7	8	9
		Our results						
No	Fluorescent compound	Concentration c_M (in mole/l)	Solvent	Layer thickness (in cm)	$\tau'_{obs.}$ (in nsec)	$\tau_{calc.}$ (in nsec) according to (3,1)		τ calc. acc. (3,10) (in nsec)
							mean v.	
1.	Fluorescein	1.10^{-6}	H_2O NaOH 1%	0,10 0,25 0,50	3,45 3,46 3,52	3,43 3,44 3,48	3,45	3,47
2.	Fluorescein	1.10^{-4}	EtOH 85% H_2O 15% 1.10^{-2}mole NaOH	0,005 0,01 0,50	3,57 3,66 3,86	3,48 3,48 3,34	3,43	3,42
3.	Fluorescein	1.10^{-4}	Glycerol 96% NaOH 1% H_2O	0,02 0,05 0,10	2,98 3,78 3,98	— — —	—	2,15
4.	Rhodamine B	1.10^{-5}	EtOH 85% H_2O+ CH_3COOH 6%	0,05 0,25 0,50	2,49 2,68 2,80	2,42 2,46 2,47	2,45	2,40
5.	Rhodamine B	1.10^{-4}	Glycerol 96%	0,10	5,17	—	—	2,94
6.	Eosine	1.10^{-4}	EtOH 85% H_2O 15% 1.10^{-2} NaOH	0,05 0,10 0,25	2,65 2,78 2,95	2,39 2,38 2,37	2,38	2,42
7.	3,6—Diaminoacridine	5.10^{-5}	EtOH 96% CH_3COOH 3%	0,05 0,10 0,25	4,09 4,23 4,29	4,02 4,09 4,05	4,05	3,89
8.	Trypaflavine	1.10^{-4}	Glycerol 96%	0,25	4,39	—	—	3,92
9.	Rhodulin orange	1.10^{-4}	Glycerol 96%	0,50	3,59	—	—	2,71
10.	Rose bengale	1.10^{-4}	EtOH 85% H_2O 15% 1.10^{-2}mole/l NaOH	0,05 0,25 0,50	0,76 0,80 0,68	— — —	—	—
11.	3-Aminophtalimide	5.10^{-3}	EtOH 96% H_2O 4%	0,005 0,50	10,8 10,9	— —	—	—
12.	3-Dimethylamino- -N-Methylphtalimide	1.10^{-2}	EtOH 96% H_2O 4%	0,005 0,50	7,29 7,11	— —	—	—
13.	3-Monomethyl- aminophtalimide	1.10^{-3}	EtOH 96% H_2O 4%	0,01 0,50	5,40 5,52	— —	—	—
14.	Esculin	1.10^{-4}	EtOH + NaOH	0,05— 0,50	3,9	—	—	—
15.	Quinine sulphate	1.10^{-5} 1.10^{-4} 1.10^{-4} 1.10^{-3} 1.10^{-3}	H_2O+ 1n H_2SO_4	0,50 0,02 0,50 0,01 0,50	18,9 18,6 19,9 18,3 18,5	— — — — —	— — — — —	— — — — —

Table I/b

10	11	12	13	14
Results in literature				
Concentration	Solvent	Layer thickness (in cm)	τ obs. (in nsec)	Authors, (year), [references]
2.10^{-6} mole/l	H_2O	thin	3,6	Schmillen (1953) [7]
1.10^{-5} mole/l	H_2O	1	4,8	Bennett (1960) [8]
1.10^{-6} mole/l	H_2O; 10^{-2} M KOH	very thin	3,83	Müller et al. (1965) [9]
—	H_2O+EtOH	—	5,07	Szymanowski (1935) [10]
2.10^{-4} g/cm³	EtOH+NaOH	1	5,5	Galanin (1950) [11]
1.10^{-5} g/g	EtOH+Glycerol	1	4,45	Bauer (1963) [12]
$1.15.10^{-4}$ g/cm³	Glycerol	1	5,13	Szymanowski (1935) [10]
3.10^{-4} mole/l	Glycerol	0,02	4,0	Galanin (1960) [13]
1.10^{-4} mole/l	Glycerol 60%	1	4,18	Hevesi et al. (1965) [14]
1.10^{-3} g/cm³	Methanol	1	2,4	Szymanowski (1935) [10]
1.10^{-4} g/cm³	EtOH+NaOH	1	3,3	Galanin (1950) [11]
1.10^{-6} mole/l	EtOH+HCl	1	4,65	Brewer et al. (1962) [15]
1.10^{-3} g/cm³	Glycerol	1	4,73	Szymanowski (1935) [10]
5.10^{-5} g/cm³	EtOH+NaOH	—	5,1	Maercks (1938) [16]
1.10^{-4} mole/l	H_2O	—	4,73	Kirchhoff (1940) [17]
$2.5.10^{-4}$ g/cm³	EtOH+NaOH	—	5,4	Galanin (1950) [11]
$2,5.10^{-4}$ g/cm³	EtOH+NaOH	—	3.4	Galanin (1950) [11]
—	H_2O	—	12,0	Sevchenko et al. (1965) [18]
—	EtOH	—	3,8	Sevchenko et al. (1965) [18]
2.10^{-3} g/cm³	EtOH	—	12,5	Galanin (1950) [11]
1.10^{-5} mole/l	1n H_2SO_4	1	20,1	Birks, Dyson (1963) [19]
$10^{-6}.10^{-4}$ mole/l	1n H_2SO_4	—	19,4	Ware-Baldwin (1964) [20]
1.10^{-5} mole/l	10^{-2} M HNO_3	2	20,8	Metcalf (1965) [21]
1.10^{-5} mole/l	2n H_2SO_4	1	22,8	Röllig (1966) [22]

in Fig. 5 as a function of the logarithm of γ_{max} ($=k_{\lambda''max} \cdot l$). The dyestuffs, concentrations and solvents are indicated in the figure; the curves *a* represent measured values, the straights *b* values corrected according to Eq. (3,1). [1]

Fig. 6 gives measured and corrected values of the decay time of fluorescein in a 1% aqueous NaOH solution for various concentrations and several layer thicknesses. Curves 1, 2 and 3 show values of τ' measured with the layer thicknesses 1 cm, 0,1 cm and 0,01 cm respectively, curve 4 the true decay times calculated with Eq. (3,1), and the points marked by filled circles values of τ calculated according to Eq. (3,10) on the base of τ' measured with three different layer thicknesses.

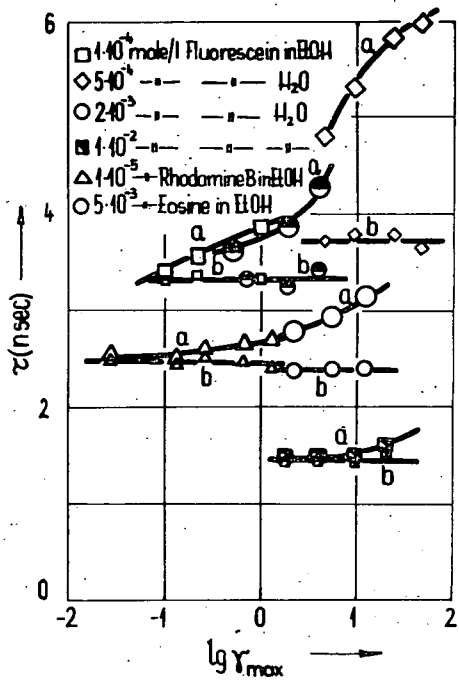

Fig. 5. Measured and true decay times of some dyestuff solutions *versus* lg γ_{max}. Curves *a:* measured values, straights *b:* values corrected according to Eq. (3,1).

6. Measured values of τ', τ-values corrected according to Eqs. (3,1) and (3,10), as well as decay times measured by different authors under similar conditions are collected in Table I.

It can be seen from the table that the deviations of the values of τ for different layer thicknesses, corrected according to Eq. (3,1), are between the limits of error of the measurements. Furthermore, the true decay times calculated with Eq. (3,10), without taking into account $\varkappa$, are in very good accordance with those corrected according to Eq. (3,1). It is to be noted that a reasonably good accordance between our uncorrected results (τ') and the decay times measured by other authors can only be found for relatively great layer thicknesses or great concentrations. In case of earlier measurements, performed with low concentrations or small layer thicknesses, a better accordance with our corrected values (τ) can be found.

For dyestuffs in which reabsorption has not been observed (quinine sulphate) or is negligibly low (esculin, phtalimides), as well as in case of small quantum yield (rose bengale, $\eta = 0{,}3$) the deviations of the measured values τ' are between the limits of error of the measurements, even if the layer thicknesses differ by two orders of magnitude.

* * *

[1] All the luminescent compounds and the solvents have been carefully purified and the solution kept at room temperature under normal atmospherical conditions during the experiments.

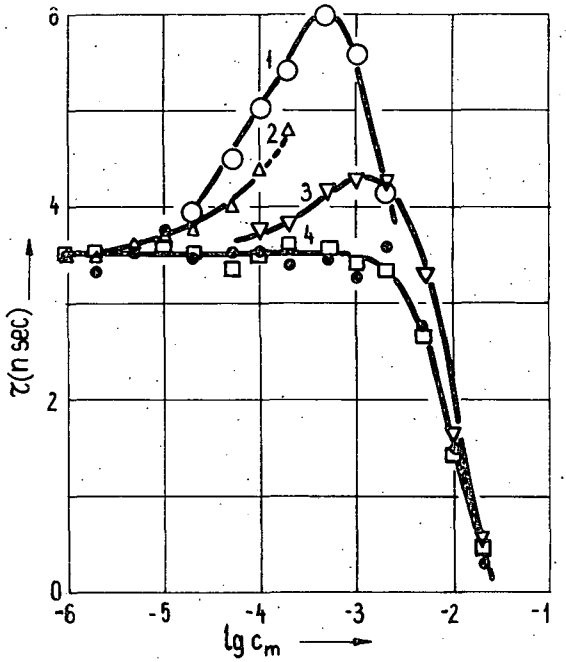

Fig. 6. Decay times of fluorescein in a 1% aqueous NaOH solution *versus* logarithm of molar concentration for different layer thicknesses l. Curve 1: $l = 1$ cm, curve 2: $l = 0{,}1$ cm, curve 3: $l = 0{,}01$ cm; curve 4: □ values corrected according to Eq. (3,1), ● values calculated with Eq. (3,10).

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ОПРЕДЕЛЕНИЕ ДЛИТЕЛНОСТИ ФЛУОРЕСЦЕНЦИИ РАСТВОРОВ В ОБЛАСТИ НАНОСЕКУНДЫ С ФАЗОВЫМ ФЛУОРОМЕТРОМ

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Написанно новый фазовый флуорометр, который содержит в себе преимущества ранее построенных приборов. Этим прибором можно измерить время затухания флуоресценции в области нсек (от 0,1-до 30 нсек) с абсолютной ошибкой $\pm 0{,}07$ нсек.

После градуировочных измерений экспериментально исследовались влияния вторичной люминесценции на время затухания и некоторыми примерами показали его важность.

Вместо сложного расчёта $\varkappa$, для которого нужно было использовать и другие характери люминесценции, задаётся простая формула, с помощью которой при заданных, экспериментально хорошо выполнимых, условиях можно определить истинное значение время затухания.